**Table 1 Kinetic parameters for ATP**

The autophosphorylation reaction was performed using the indicated enzyme and gefitinib (0.5–5 nM). The steady-state kinetic parameters for ATP were determined from the Eadie–Hofstee plot in Figure 5. Results are means ± S.D. for three independent duplicate experiments.

| | | $K_m$ ($\mu$M) | | | | $V_{max}$ ($\mu$M · min$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Deletion mutant | | Wild-type | | Deletion mutant | | Wild-type | |
| Gefitinib (nM) | EGF stimulation... | − | + | − | + | − | + | − | + |
| 0 | | 2.5 ± 0.2 | 2.2 ± 0.2 | 4.0 ± 0.3 | 1.9 ± 0.1 | 1.9 ± 0.1 | 2.1 ± 0.1 | 2.0 ± 0.0 | 1.9 ± 0.0 |
| 0.5 | | 5.6 ± 0.5 | 5.7 ± 0.4 | 4.1 ± 0.4 | 2.3 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.2 | 2.0 ± 0.1 | 1.9 ± 0.1 |
| 1 | | 9.8 ± 2.8 | 10.9 ± 3.0 | 4.6 ± 1.2 | 2.5 ± 0.1 | 2.0 ± 0.1 | 1.9 ± 0.0 | 2.0 ± 0.2 | 1.8 ± 0.1 |
| 5 | | 26.1 ± 5.4 | 30.2 ± 4.2 | 7.0 ± 2.3 | 4.9 ± 0.9 | 1.9 ± 0.1 | 1.8 ± 0.2 | 2.0 ± 0.1 | 1.8 ± 0.2 |

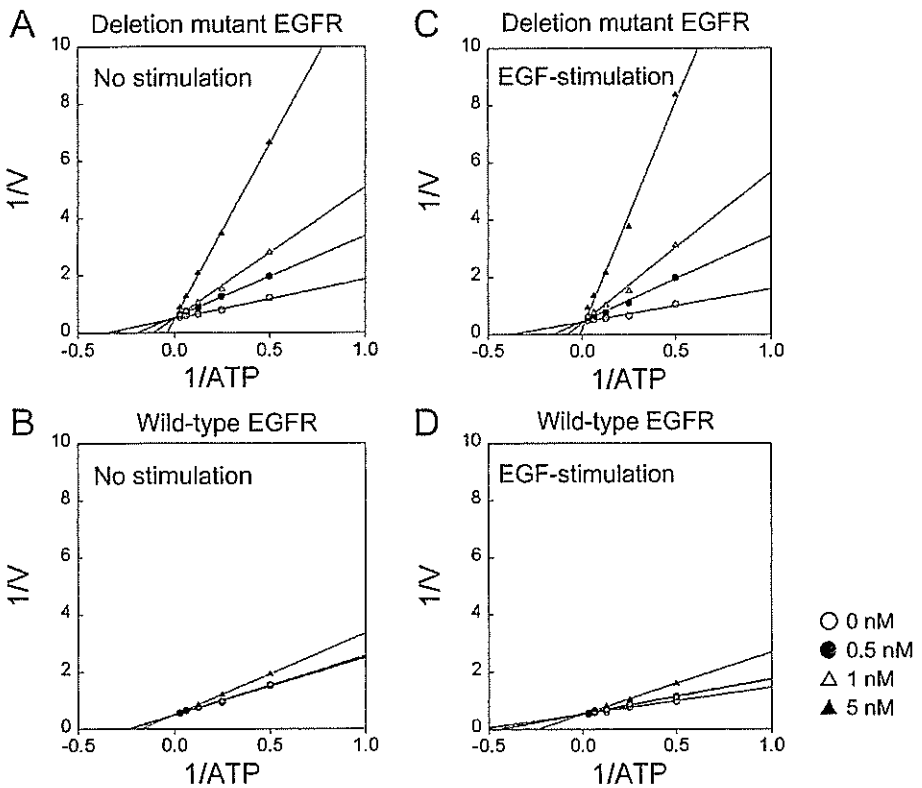


**Figure 4 Mechanism of inhibition of deletion mutant EGFR by gefitinib**

Autophosphorylation of unstimulated deletion mutant (**A**), unstimulated wild-type (**B**), EGF-stimulated deletion mutant (**C**) and EGF-stimulated wild-type (**D**) EGFR was measured with or without gefitinib at concentrations of 0 (○), 0.5 (●), 1 (△) and 5 (▲) nM. Reciprocal velocity against reciprocal ATP concentrations (0.5–32 $\mu$M) were plotted. Data are representative of at least three independent experiments.

low level of EGF-independent basal phosphorylation, whereas autophosphorylation using EGF-stimulated EGFR represents EGF-induced phosphorylation.

### Kinetic parameters of autophosphorylation

The deletion mutant EGFR is constitutively phosphorylated under unstimulated conditions. Measuring the autophosphorylation activity of deletion mutant EGFR requires unphosphorylated tyrosine residues of EGFR. An autophosphorylation assay was reconstructed to determine the kinetic parameters of deletion mutant EGFR. The method is summarized in Figure 2. The concentrations of gefitinib used (2 $\mu$M) completely inhibited phosphorylation of both the deletion mutant and wild-type EGFR, as demonstrated by immunoblot analysis (Figure 1C). We performed autophosphorylation assays with various amounts of EGFR (results not shown). In our autophosphorylation assay, a constant amount of EGFR (130 ng/well) was adopted to measure its autophosphorylation, because this amount of EGFR was found to be appropriate for detecting changes in the absorbance of both wild-type and deletion mutant EGFR. The autophosphorylation of deletion mutant EGFR and wild-type EGFR was analysed by comparison with unstimulated and EGF-stimulated EGFR (Figure 3). The higher phosphorylation of deletion mutant EGFR shown in Figure 1(A) was lowered by using gefitinib-treated lysates, while the autophosphorylation reaction was initiated by addition of ATP. The ATP-dependent autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR in crude cellular extracts were monitored (Figure 3, insets). The data were transformed into an Eadie–Hofstee plot, and the kinetic parameters were determined as apparent $K_m$ and $V_{max}$ values for ATP (Figure 3 and Table 1). Under unstimulated conditions,

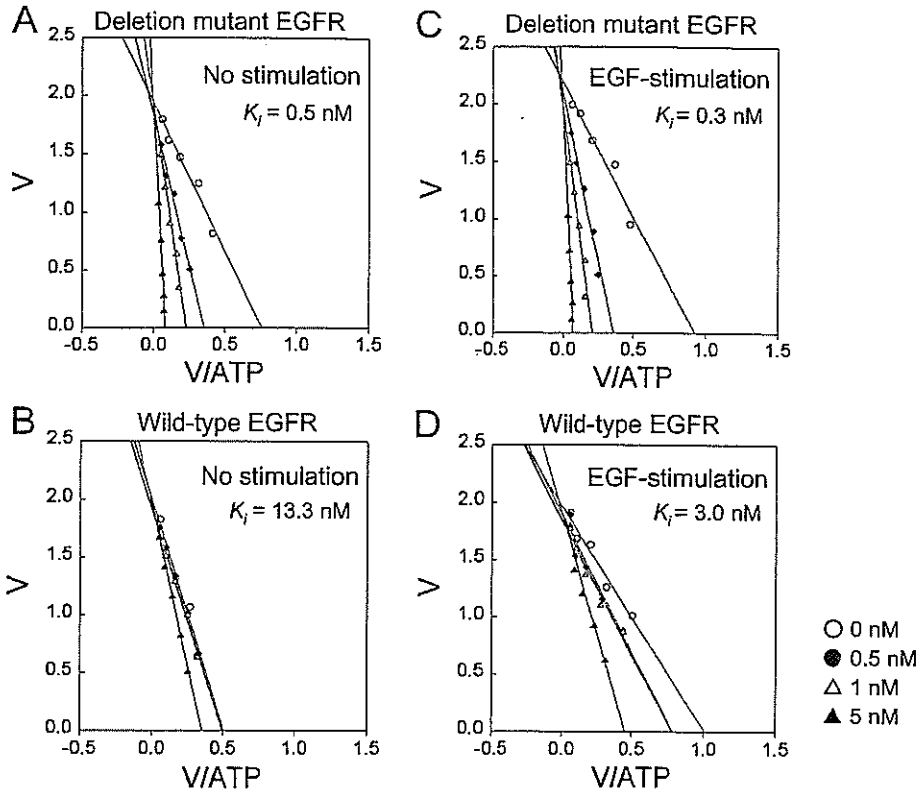


**Figure 5 Inhibition constant of gefitinib for autophosphorylation activity of deletion mutant EGFR**

The same dataset as shown in Figure 4 was fitted to an Eadie–Hofstee plot, and kinetic parameters from this fit are summarized in Table 1. Shown are the results for the unstimulated (**A**) and EGF-stimulated (**C**) deletion mutant EGFR and unstimulated (**B**) and EGF-stimulated (**D**) wild-type EGFR in response to ATP with or without gefitinib at concentrations of 0 (○), 0.5 (●), 1 (△) and 5 (▲) nM. Results are representative of at least three independent experiments.

differences in activities were seen between unstimulated wild-type ($K_m$ for ATP = $4.0 \pm 0.3$ $\mu$M) and deletion mutant EGFR ($K_m$ for ATP = $2.5 \pm 0.2$ $\mu$M). Under EGF-stimulated conditions, there was no difference in $K_m$ values between EGF-stimulated wild-type EGFR ($K_m$ for ATP = $1.9 \pm 0.1$ $\mu$M) and deletion mutant EGFR ($K_m$ for ATP = $2.2 \pm 0.2$ $\mu$M). The $V_{max}$ values of wild-type EGFR and deletion mutant EGFR were equal under both conditions. These results suggest that the wild-type EGFR is conformationally activated by EGF stimulation, and that the mutant EGFR is active without ligand stimulation.

### Gefitinib inhibits autophosphorylation of deletion mutant EGFR

We examined the inhibitory effect of gefitinib (0.5, 1 and 5 nM) on the autophosphorylation of deletion mutant EGFR in comparison with wild-type EGFR under unstimulated and EGF-stimulated conditions. The data were transformed into a Lineweaver–Burk plot for estimation of the mode of inhibition (Figure 4). Lineweaver–Burk plot analysis showed that gefitinib competitively inhibited the autophosphorylation of deletion mutant EGFR as well as that of wild-type EGFR. The data were transformed into an Eadie–Hofstee plot for determination of kinetic parameters (Figure 5). Eadie–Hofstee plot analysis revealed the apparent $K_m$ and $V_{max}$ values for ATP in the presence of various gefitinib concentrations, and the kinetic parameters are summarized in Table 1. The $K_i$ for deletion mutant EGFR and wild-type EGFR was calculated using eqn 1 (see the Materials and methods section). The $K_i$ value of gefitinib for deletion mutant EGFR ($K_i$ for gefitinib = $0.5 \pm 0.1$ nM) was 26-fold lower than that for wild-type EGFR ($K_i$ for gefitinib = $13.3 \pm 5.1$ nM) under unstimulated conditions (Figure 5). Under EGF-stimulated conditions, the $K_i$ value of gefitinib for deletion mutant EGFR ($0.3 \pm 0.1$ nM) was 10-fold lower than that for wild-type EGFR ($3.0 \pm 0.6$ nM) (Figure 5). Based on these comparative studies, we concluded that gefitinib binds deletion mutant EGFR more strongly than wild-type EGFR. In addition, we calculated the inhibitory effect of gefitinib for both types of EGFR in the presence of 2 $\mu$M ATP (Figure 6). Relatively strong inhibitory activity was detected for deletion mutant EGFR as compared with wild-type EGFR. These results suggest that gefitinib had a high affinity (low $K_i$ value) for deletion mutant EGFR compared with wild-type EGFR.

## DISCUSSION

Wild-type EGFR is unphosphorylated, being in an inactive form, under unstimulated conditions. The binding of ligands to the extracellular domain of EGFR induces dimerization and phosphorylation of the receptor into the active form [13]. The kinetic parameters of wild-type EGFR in our autophosphorylation assay are consistent with those of previous reports [14,15]. Crystallographic analysis has shown that the structure of the EGFR kinase domain after forming a complex with erlotinib exhibits a conformation consistent with the active form of protein kinases [16,17]. Previously, we reported that the deletion mutant EGFR was dimerized and phosphorylated constitutively without ligand stimulation, suggesting an active conformation [9]. We analysed the enzymatic properties of the deletion mutant EGFR, and

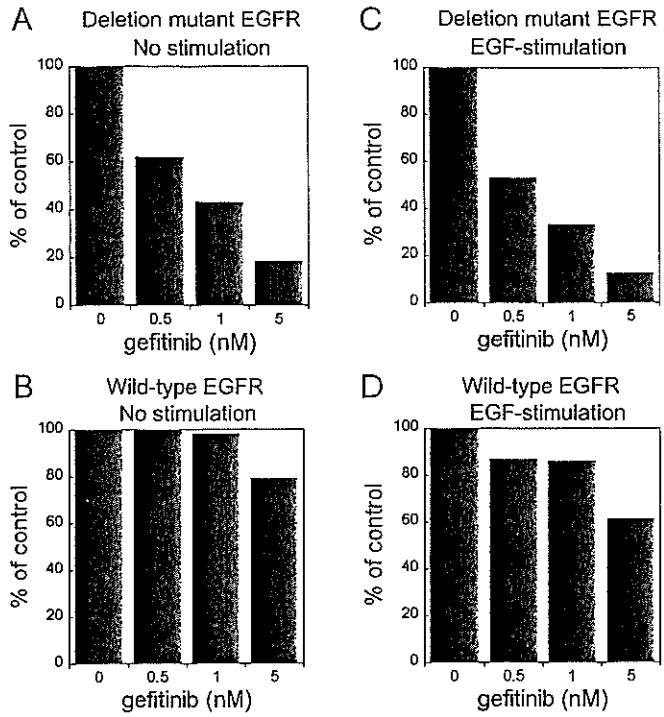


**Figure 6 Effects of gefitinib on autophosphorylation of deletion mutant EGFR**

The percentage of absorbance compared with the control under conditions of 2 $\mu$M ATP was calculated using the same dataset as shown in Figure 4 at a concentration of 2 $\mu$M ATP. The results shown are for unstimulated (**A**) and EGF-stimulated (**C**) deletion mutant EGFR and unstimulated (**B**) and EGF-stimulated (**D**) wild-type EGFR in response to ATP with or without gefitinib. Results are representative of at least three independent experiments.

determined the $K_i$ value of gefitinib for deletion mutant EGFR. The inhibition constant of gefitinib for wild-type EGFR was similar to the value reported by Wakeling et al. [18]. We showed that the $K_i$ value of gefitinib for deletion mutant EGFR was much lower than that for wild-type EGFR. The evidence of the decreased $K_i$ value of gefitinib for deletion mutant EGFR means that gefitinib binds deletion mutant EGFR more strongly than wild-type EGFR. The high-affinity interaction between deletion mutant EGFR and gefitinib may be attributable to structural differences between deletion mutant EGFR and wild-type EGFR.

Our conclusion does not contradict the previous report by Stamos et al. [16] on a similar EGFR-targeted tyrosine kinase inhibitor, erlotinib, which binds to the active form of EGFR [14]. This result differs from that reported elsewhere: Fabian et al. [19] reported that there were no differences in the binding affinity of EGFR-targeted tyrosine kinase inhibitors between wild-type EGFR and mutant EGFR, including the deletion mutation. They constructed and expressed the kinase domain of EGFR on a bacteriophage surface, followed by interaction with immobilized inhibitors using biotin–avidin systems. Conversely, in our experiments, we performed autophosphorylation assays with EGFR extracted from 293-p$\Delta$15 and the 293-pEGFR cells overexpressing deletion mutant and wild-type EGFR respectively. We consider our cell-based autophosphorylation assay results to reflect the native state of deletion mutant EGFR and to possibly explain the hypersensitivity of mutant-expressing cells to gefitinib.

We demonstrated that the deletion mutant actually binds gefitinib more strongly than wild-type EGFR. This is likely to be the mechanism of action of other tyrosine kinase inhibitors such as erlotinib, ZD6474 [dual inhibitor targeted to VEGFR2 (vascular endothelial growth factor receptor 2)/KDR (kinase insert domain-containing receptor) and EGFR] and other possible multi-targeted tyrosine kinase inhibitors. Indeed, EGFR-specific tyrosine kinase inhibitors AG1478 and erlotinib, as well as ZD6474, as described in our previous report [7] showed different growth-inhibitory activities against HEK-293 transfected with deletion mutant EGFR (results not shown). Thus it is likely that these (ATP competitive) tyrosine kinase inhibitors have different binding property effects on wild-type and deletion mutant EGFR to those of gefitinib.

In the present study, we focused on the enzymatic properties of in-frame deletion mutant EGFR (delE746–A750). The inhibition of receptor autophosphorylation in deletion mutant EGFR by gefitinib was much greater than that in wild-type EGFR. Next, it is necessary to examine the kinetic properties of other types of EGFR mutants, especially L858R, and these findings may pave the way for the discovery of different kinase inhibitors with different inhibition profiles for EGFR.

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## REFERENCES

1 Arteaga, C. L. (2003) ErbB-targeted therapeutic approaches in human cancer. Exp. Cell Res. **284**, 122–130
2 Traxler, P., Furet, P., Mett, H., Buchdunger, E., Meyer, T. and Lydon, N. (1997) Design and synthesis of novel tyrosine kinase inhibitors using a pharmacophore model of the ATP-binding site of the EGF-R. J. Pharm. Belg. **52**, 88–96
3 Shepherd, F. A., Rodrigues Pereira, J., Ciuleanu, T., Tan, E. H., Hirsh, V., Thongprasert, S., Campos, D., Maoleekoonpiroj, S., Smylie, M., Martins, R. et al. (2005) Erlotinib in previously treated non-small-cell lung cancer. N. Engl. J. Med. **353**, 123–132
4 Bell, D. W., Lynch, T. J., Haserlat, S. M., Harris, P. L., Okimoto, R. A., Brannigan, B. W., Sgroi, D. C., Muir, B., Riemenschneider, M. J., Iacona, R. B. et al. (2005) Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J. Clin. Oncol. **23**, 8081–8092
5 Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G. et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. **350**, 2129–2139
6 Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J. et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science **304**, 1497–1500
7 Arao, T., Fukumoto, H., Takeda, M., Tamura, T., Saijo, N. and Nishio, K. (2004) Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res. **64**, 9101–9104
8 Tracy, S., Mukohara, T., Hansen, M., Meyerson, M., Johnson, B. E. and Janne, P. A. (2004) Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. Cancer Res. **64**, 7241–7244
9 Sakai, K., Arao, T., Shimoyama, T., Murofushi, K., Sekijima, M., Kaji, N., Tamura, T., Saijo, N. and Nishio, K. (2005) Dimerization and the signal transduction pathway of a small in-frame deletion in the epidermal growth factor receptor. FASEB J. **20**, 311–313
10 Nishio, K., Arioka, H., Ishida, T., Fukumoto, H., Kurokawa, H., Sata, M., Ohata, M. and Saijo, N. (1995) Enhanced interaction between tubulin and microtubule-associated protein 2 via inhibition of MAP kinase and CDC2 kinase by paclitaxel. Int. J. Cancer **63**, 688–693
11 Kawamura-Akiyama, Y., Kusaba, H., Kanzawa, F., Tamura, T., Saijo, N. and Nishio, K. (2002) Non-cross resistance of ZD0473 in acquired cisplatin-resistant lung cancer cell lines. Lung Cancer **38**, 43–50
12 Koizumi, F., Shimoyama, T., Taguchi, F., Saijo, N. and Nishio, K. (2005) Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. Int. J. Cancer **116**, 36–44
13 Tanner, K. G. and Kyte, J. (1999) Dimerization of the extracellular domain of the receptor for epidermal growth factor containing the membrane-spanning segment in response to treatment with epidermal growth factor. J. Biol. Chem. **274**, 35985–35990
14 Nair, N., Davis, R. J. and Robinson, H. L. (1992) Protein tyrosine kinase activities of the epidermal growth factor receptor and ErbB proteins: correlation of oncogenic activation with altered kinetics. Mol. Cell. Biol. **12**, 2010–2016

15 Wood, E. R., Truesdale, A. T., McDonald, O. B., Yuan, D., Hassell, A., Dickerson, S. H., Ellis, B., Pennisi, C., Horne, E., Lackey, K. et al. (2004) A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. Cancer Res. **64**, 6652–6659
16 Stamos, J., Sliwkowski, M. X. and Eigenbrot, C. (2002) Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J. Biol. Chem. **277**, 46265–46272

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Published as BJ Immediate Publication 20 April 2006, doi:10.1042/BJ20051962

17 Noble, M. E., Endicott, J. A. and Johnson, L. N. (2004) Protein kinase inhibitors: insights into drug design from structure. Science **303**, 1800–1805
18 Wakeling, A. E., Guy, S. P., Woodburn, J. R., Ashton, S. E., Curry, B. J., Barker, A. J. and Gibson, K. H. (2002) ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res. **62**, 5749–5754
19 Fabian, M. A., Biggs, 3rd, W. H., Treiber, D. K., Atteridge, C. E., Azimioara, M. D., Benedetti, M. G., Carter, T. A., Ciceri, P., Edeen, P. T., Floyd, M. et al. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. Nat. Biotechnol. **23**, 329–336

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# Dimerization and the signal transduction pathway of a small in-frame deletion in the epidermal growth factor receptor

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## SPECIFIC AIM

A short, in-frame deletional mutant (E746-A750del) a major mutant form of EGFR in non-small cell lung cancer, and has been reported to be a major determinant of response to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib. However, the biological and pharmacological functions of mutational EGFR remain unclear. The aim of this study is to clarify whether it is constitutively active or not and whether alteration occurs downstream of the intracellular signaling.

## PRINCIPAL FINDINGS

### 1. A short, in-frame deletional mutant (E746-A750del) induced dimerization and phosphorylation of EGFR without any ligand stimulation

To determine the biological functions of deletion mutant (E746-A750del) EGFR, we used the stable transfected cells of wild-type and deletion mutant of EGFR. Previously, we demonstrated that the 293(D) cells transfected with the deletional EGFR were hypersensitive to EGFR-targeted tyrosine kinase inhibitors such as gefitinib and ZD6474 as compared with the 293(W) cells transfected with wild-type EGFR. Dimerization and phosphorylation of EGFR in these cells were determined by using chemical cross-linker and by immunoblot analysis (**Fig. 1**). No expression of EGFR dimer or monomer was detected in the 293(M) cells. Increased dimerization and phosphorylation of the deletional EGFR with a molecular weight of ~400 kDa were detected without EGF stimulation in the 293(D) cells. When stimulated with the EGF, increased dimerized and phosphorylated EGFR were observed in the 293(W) cells, whereas no response of EGFR to EGF was observed in the 293(D) cells. The ratio of dimerized to monomeric EGFR in 293(W) and 293(D) cells was analyzed densitometrically (Fig. 1, right). The dimer/monomer ratio in the 293(W) cells was markedly increased (~3-fold) by addition of EGF. Under unstimulated conditions, the dimer/monomer ratio of the 293(D) cells was higher than that of the 293(W) cells and the ratio was unchanged by addition of EGF. These results suggest that the cells expressing the wild-type of EGFR responded to EGF for their dimerization and phosphorylation and that the deletional mutant of EGFR was dimerized and phosphorylated constitutively without any ligand stimulation.

### 2. p44/42 MAPK and AKT pathways are activated in the cells expressing deletional EGFR without ligand stimulation

We examined the phosphorylation status of p44/42 MAPK and AKT that are major downstream targets of EGFR in the transfectants. Even under unstimulated conditions, increased phosphorylation of p44/42 MAPK and AKT was observed in the 293(D) cells. In the 293(W) cells, increased phosphorylation of p44/42 MAPK and AKT was observed with the addition of EGF but p44/42 MAPK was remarkably phosphorylated. On the other hand, no increased phosphorylation of p44/42 MAPK and AKT was observed with the addition of EGF in the 293(D) cells. This result suggests that the p44/42 MAPK and AKT pathways are activated in cells expressing the deletional EGFR without ligand stimulation.

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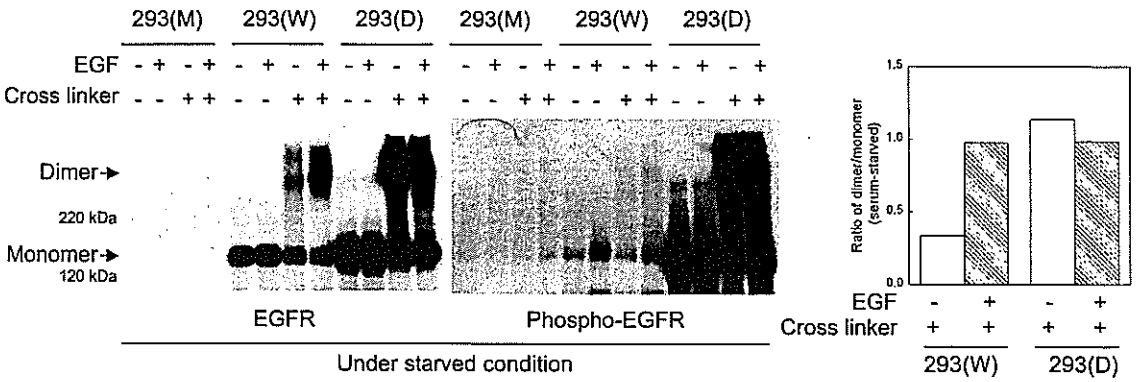


**Figure 1.** Dimerization and phosphorylation of wild-type EGFR and deletional EGFR A. The 293 cells transfected with the empty vector (293(M)), wild-type EGFR (293(W)), and deletional EGFR (293(D)) were treated with or without EGF (10 ng/mL) for 10 min after serum starvation. After two washes with ice-cold PBS(+), monolayer cells were incubated with the chemical cross-linking reagent $BS^3$ (1.5 mM) in PBS(+). Glycine (20 mM) was added for an additional 5 min to terminate the reaction. The lysates (twenty μg protein) were subjected to 2–15% SDS-PAGE followed by immunoblot analysis using anti-EGFR and anti-phospho-EGFR. Right panel: ratio of dimerized to monomeric EGFR.

### 3. Gefitinib inhibited the AKT signaling pathway more strongly than the p44/42 MAPK signaling pathway

We next determined the action of EGFR-targeted tyrosine kinase inhibitor gefitinib on downstream of deletional EGFR (**Fig. 2A**). In the 293(W) cells, phosphorylation of p44/42 MAPK was not inhibited by exposure to a low dose of gefitinib (0.01 μM) but phosphorylation of AKT was inhibited by exposure to gefitinib (~70%, Fig. 2*C*). In contrast, exposure to gefitinib decreased phospho-EGFR in the 293(D) cells. Phosphorylation of AKT was completely inhibited by 0.01 μM gefitinib exposure (~99%, Fig. 2*C*), whereas inhibition of p44/42 MAPK phosphorylation was not remarkable in the 293(D) cells (~20%, Fig. 2*B*). These data suggest that gefitinib inhibited the AKT signaling pathway more strongly than the p44/42 MAPK signaling pathway in the cells expressing the deletion mutant EGFR.

### 4. AKT pathway was activated in the PC-9 cells expressing deletional EGFR intrinsically

To examine whether increased phosphorylation is also observed in the lung cancer cells intrinsically expressing deletional EGFR, we monitored the phosphorylation of EGFR and its related molecules in the PC-9 cells expressing deletional EGFR by using a beads-based mulitiplex assay. We found increased phosphorylation of EGFR and downstream molecules of AKT pathway including IκB-α in PC-9 cells. This finding is consistent with the result of the previous experiments with the 293(D) cells. It is suggested that AKT pathway is activated in the cells expressing deletional EGFR intrinsically.

## CONCLUSIONS AND SIGNIFICANCE

To clarify the function of deletional EGFR, we used the cell transfectants with deletional EGFR [293(D)] that is

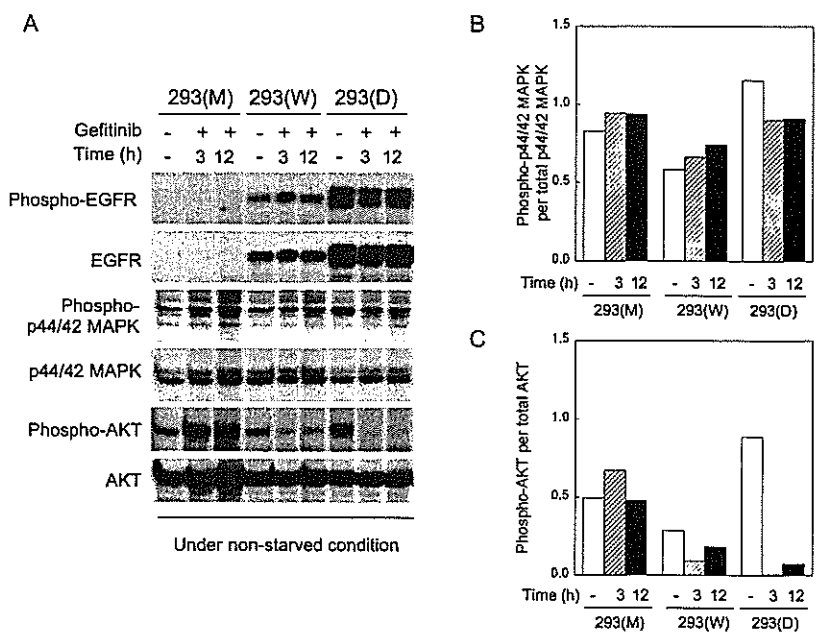


**Figure 2.** Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR transfected 293 cells. *A)* The 293(M), 293(W), and 293(D) cells were incubated with gefitinib (0.01 μM) for 3 h or 12 h under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, and phospho-AKT, then subjected to immunoblot analysis. *B)* Histogram of the degree of p44/42 MAPK activation expressed as phospho-p44/42 MAPK per total p44/42 MAPK. *C)* Histogram of the degree of AKT activation expressed as phospho-AKT per total AKT.

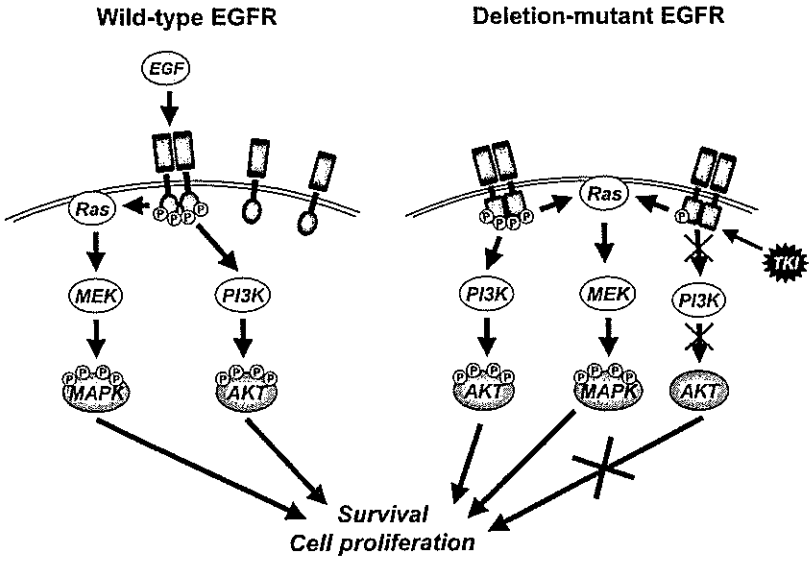


**Figure 3.** Function of deletional EGFR. Wild-type EGFR is dimerized and phosphorylated by EGF the wild-type EGFR and MAPK and AKT pathways are activated. The deletion mutant EGFR is dimerized and phosphorylated without EGF. Both MAPK and AKT pathways are activated; but phospho-AKT was inhibited by TKI predominantly in the cells expressing deletional EGFR. MEK, MAP kinase/extracellular regulated kinases; PI3K, phosphoinositide-3-kinase; TKI, tyrosine kinase inhibitors.

hypersensitive to tyrosine kinase inhibitors (e.g., gefitinib). We detected significantly higher levels of dimerization and phosphorylation of deletional EGFR without any ligand stimulation in the cells deletional EGFR. Increased phosphorylation of p44/42 MAPK and AKT was observed in the 293(D) cells. These results suggest that deletional EGFR is constitutively active. When the 293(D) cells were exposed to gefitinib (0.01 μM), AKT phosphorylation was completely suppressed, suggesting that deletional EGFR signaling inclines toward the AKT pathways. A summary of characteristics of deletional EGFR is shown in **Fig. 3.**

An additional experiment using a PC-9 lung cancer cell line intrinsically expressing deletional EGFR confirmed the gain of function of deletional EGFR and activated AKT signaling pathway.

Results from this study have provided the understanding for biological functions of deletional EGFR and cellular hypersensitivity to the EGFR-targeted tyrosine kinase inhibitor.

Now over 30 types of mutation have been reported in clinical lung cancer specimens. We will examine the biological function of other types of EGFR mutants differentially, with the aim of selecting clinically meaningful mutations. FJ

# *EGFR* Mutation of Tumor and Serum in Gefitinib-Treated Patients with Chemotherapy-Naive Non–small Cell Lung Cancer

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**Background:** The authors evaluate the efficacy and safety of gefitinib monotherapy in chemotherapy-naive patients with advanced non–small-cell lung cancer (NSCLC). A secondary endpoint is to evaluate the relationship between clinical manifestations and epidermal growth factor receptor (*EGFR*) mutation status.

**Methods:** Japanese chemotherapy-naive NSCLC patients were enrolled. They had measurable lesions, Eastern Cooperative Oncology Group performance status of 0 to 2, and adequate organ and bone marrow function. Patients received 250 mg of oral gefitinib daily. *EGFR* mutations in exon 18, 19, and 21 of DNA extracted from tumor and serum were analyzed by genomic polymerase chain reaction and direct sequence.

**Results:** All 30 patients were eligible for the assessment of efficacy and safety. An objective response and stable disease were observed in 10 patients (33.3%) and nine patients (30.0%), respectively. The median time to progression was 3.3 months and the median overall survival was 10.6 months. The 1-year survival rate was 43.3%. Grade 3 toxicities were observed in seven patients. *EGFR* mutation was observed in four of 13 (30.8%) tumors, and two of them achieved partial response. In serum samples, three of 10 patients with *EGFR* mutations in the serum before treatment had a response to gefitinib. *EGFR* mutation was observed in 10 of 27 and significantly more frequently observed in the posttreatment samples from patients with a partial response or stable disease than in those from patients with progressive disease ($p = 0.006$).

**Conclusions:** Gefitinib monotherapy in chemotherapy-naive NSCLC patients was active, with acceptable toxicities. These results warrant further evaluation of gefitinib monotherapy as a first-line therapy. The *EGFR* mutation in serum DNA may be a biomarker for monitoring the response to gefitinib during treatment.

**Key Words:** Non-small-cell lung cancer, Gefitinib, Epidermal growth factor receptor, Mutation, Serum DNA.

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Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death in Japan and throughout the world.[1] Unfortunately, the majority of patients with NSCLC present with locally advanced or metastatic disease at the time of diagnosis. Although chemotherapy has produced modest survival benefits in advanced NSCLC patients, the outcome of chemotherapy for NSCLC remains unsatisfactory.

Protein tyrosine kinases play important roles in the pathogenesis of malignant tumors.[2] Among them, epidermal growth factor receptor (EGFR) tyrosine kinase has been implicated in the initiation and progression of NSCLC.[3–5] The overexpression of EGFR is frequent in NSCLC.[6] Monoclonal antibodies and low-molecular-weight compounds that inhibit the EGFR signaling pathway have been developed and shown to have antitumor effects. Gefitinib (Iressa, AstraZenca, London, England) is an orally active EGFR type tyrosine kinase inhibitor. In four phase I studies, tumor shrinkage or stabilization after gefitinib monotherapy was observed in some patients with NSCLC. In two phase II trials, Iressa Dose Evaluation in Advanced Lung cancer (IDEAL) 1 and 2, gefitinib monotherapy was shown to have a substantial effect in NSCLC patients treated previously with chemotherapy.[7,8] In these trials, patients of Asian origin and who had never been smokers had a statistically significant improvement in overall survival. In spite of encouraging results in the IDEAL trials, two large-scale, phase III, randomized trials, Iressa NSCLC Trial Assessing Combination Treatment, failed to show any survival benefit for the use of gefitinib.[9,10] Patients in a large-scale phase III trial comparing gefitinib

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and placebo in advanced NSCLC with prior chemotherapy demonstrated in preliminary analysis a tendency to have improvement in overall survival but did not have a statistically significant improvement in overall survival.[11] There are many issues that need to be addressed with regard to the clinical application of gefitinib: one of the most important issues is the efficacy of gefitinib monotherapy in patients with chemotherapy-naive NSCLC,[12] and another is to establish a way to predict response to gefitinib.

Recently, it has been suggested that mutations in the EGFR tyrosine kinase domain play a critical role in determining tumor response to gefitinib in NSCLC patients.[13,14] The mutations consisted of small, in-frame deletions or substitutions clustered around the adenosine triphosphate-binding site in exons 18, 19, and 21 of the *EGFR*. After these reports, some investigators supported the belief that *EGFR* mutation is one of the strong determinants of tumor response to gefitinib.[15–17] Tumors with *EGFR* mutations tend to be more common in adenocarcinomas, female patients, nonsmokers, and those of Asian origin. In most of those studies, tumor samples that were resected by operations were used. Because it is often difficult to obtain a tumor sample from an inoperable NSCLC patient, it is necessary to establish a method for detecting mutant *EGFR* from a patient sample other than from tumor specimens.

Polymerase chain reaction (PCR) technology for the amplification of small amounts of DNA has made it possible to identify the same alterations typically observed in DNA from serum samples from NSCLC patients.[18,19] Serum DNA may provide a noninvasive and repeatable source of genotypic information that could influence treatment and prognosis, especially in advanced NSCLC patients who have received gefitinib therapy. We essentially consider that it is possible to detect the *EGFR* mutation in serum DNA. We hypothesized that serum DNA may provide useful information on *EGFR* mutations in lung cancer patients.

As described above, the usefulness of gefitinib monotherapy is controversial and that in patients without pretreatment is unclear. Because *EGFR* mutations have been shown to be strongly associated with the response of NSCLC patients to gefitinib treatment, the analysis of *EGFR* mutations is necessary to evaluate the clinical benefit of gefitinib. We therefore conducted a multicenter phase II trial for these patients. The primary objective was to evaluate the objective response rate, and secondary objectives were to estimate the disease control rate, disease-related symptom improvement rate, safety, time to progression (TTP), and overall survival (OS). In addition, as a correlative study, we planned to detect *EGFR* mutations in serum samples from NSCLC patients and evaluate the relationship between the *EGFR* mutation and clinical manifestations in NSCLC patients receiving gefitinib treatment.

## PATIENTS AND METHODS

### Patient Eligibility

Patients who had histologically or cytologically proven stage IIIb or IV NSCLC and no previous chemotherapy were enrolled into this trial. Radiotherapy for metastatic lesions until 3 weeks before entry was allowed on condition that these lesions were not assessed for tumor response. Patients in whom recurrence occurred after surgery were also eligible. Patient eligibility criteria included at least one measurable lesion, age of 20 years or older, Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, and life expectancy of greater than or equal to12 weeks. Adequate organ and bone marrow function was necessary, defined as leukocyte counts greater than or equal to $3.0 \times 10^6$/liter, neutrophil counts greater than or equal to $1.5 \times 10^6$/liter, platelet counts greater than or equal to $100 \times 10^9$/liter, hemoglobin levels greater than or equal to 8.5 g/dl, alanine aminotransferase or aspartate aminotransferase levels less than or equal to two times the upper limit of the reference range (<100 IU/liter in the presence of liver metastases), serum bilirubin levels less than or equal to 1.5 mg/dl, serum creatinine levels less than or equal to1.5 mg/dl, and $PaO_2$ levels greater than or equal to 65 mmHg. Patients with any of the following were excluded: active double cancer; severe complications such as myocardial infarction within 3 months before entry or uncontrolled diabetes; symptomatic brain or bone metastasis; diarrhea more severe than grade 2 according to National Cancer Institute Common Toxicity Criteria version 2; systemic administration of steroids to treat skin diseases; pleural, pericardial, or peritoneal effusion requiring treatment; and pregnancy or lactation. All patients were required to give informed consent.

### Treatment

Patients were treated with gefitinib 250 mg orally once per day. Treatment was discontinued when the disease progressed, intolerable toxicities appeared, the patients requested withdrawal, or disease-related symptoms worsened without tumor response after 8 weeks of gefitinib monotherapy. These patients received chemotherapeutic treatment after gefitinib therapy. The chemotherapy regimen consisted of platinum (cisplatin or carboplatin) plus new agents (paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan) in patients aged 74 years or younger and vinorelbine monotherapy in patients aged 75 years or older. If symptomatic bone or brain metastasis occurred during gefitinib monotherapy, patients received radiotherapy after gefitinib treatment.

### Efficacy and Drug-Related Adverse Events

Tumor size was assessed with computed tomography or magnetic resonance imaging scans every 4 weeks from the start to cessation of protocol treatment, using Response Evaluation Criteria in Solid Tumors guidelines.[20] Disease control was judged when patients achieved the best response of complete response, partial response (PR), or stable disease (SD), which was confirmed and sustained for 4 weeks. TTP was measured as the period from the start of the treatment to an identifiable time of disease progression. OS was measured from the start of the treatment until death or the last follow-up. The Kaplan-Meier method was used to calculate these measures.

Drug adverse events were recorded and graded according to National Cancer Institute Common Toxicity Criteria

version 2.0. Changes in physical and laboratory findings were assessed at least every 2 weeks.

### Serum Sample Collection and DNA Extraction

Blood samples from patients were collected before and 14 days after the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. DNA extraction from the serum samples was performed using a nonorganic method (Oncor, Gaithersburg, MD). Serum DNA was purified using Qiamp Blood Kit (Qiagen, Hilden, Germany), with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The extracted DNA was stocked at −20°C until use.

### Tissue Sample Collection and DNA Extraction

Tumor specimens were obtained on protocols approved by the institutional review board. Twenty paraffin blocks of tumor material, obtained from 15 patients for diagnosis before treatment, were collected retrospectively. Eleven tumor samples were collected from primary cancer by means of transbronchial lung biopsy, one was resected by operation, and nine were from metastatic sites (four from bone, three from lymph nodes, one from the brain, and one from the colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was performed using the TaKaRa DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

### PCR Amplification

PCR was performed in 25-μl volumes using 15 μl of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μl of PCR buffer, 0.8 mM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. A set of designed primers was used to amplify exon 19 of *EGFR* (upper primer, 5′-CAGCCCCCAGCAATATCAGCCTTAGGT-3′; lower primer, 5′- CACTAGAGCTAGAAAGGGAAAGACATA-3′). Thirty cycles of amplification were performed using a thermal cycler (Perkin-Elmer, Foster City, CA) (95°C for 45 seconds, 55.5°C for 30 seconds, 72°C for 30 seconds, followed by incubation at 72°C for 10 minutes). The bands were visualized using a 2100 bioanalyzer, DNA 500 Labchip kit (Aglient Technologies, Waldbronn, Germany). If no PCR products were detected by the first PCR, an additional 20 cycles of PCR was carried out and the sample was revisualized. To confirm the deletional mutation in exon 19, and to detect the mutation in exons 18 and 21 of *EGFR*, PCR was performed again using another primer set as described previously.[13]

### Sequencing

Amplification and sequencing were performed in duplicate for each sample using an ABI prism 310 (Applied Biosystems). The sequences were compared with the GenBank-archived human sequence for *EGFR* (accession no. AY588246).

### Trial Design and Statistical Methods

The trial was a two-stage multicenter phase II study. The primary endpoint was response rate, and secondary endpoints were disease control rate, safety, TTP, and OS. As a correlative study, *EGFR* mutations in tumor and serum samples were analyzed. The protocol and consent form were approved by the institutional review board of each participating hospital. Initially, 15 patients were recruited to the study. If one of these patients responded to treatment with gefitinib monotherapy, an additional 10 patients were recruited. If five or more of these 25 patients responded to therapy, treatment with gefitinib was concluded to be effective. According to Simon's minimax design,[21] our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was greater than 30% and a 5% significance to deny the hypothesis that the true objective response rate was less than 10%. Assuming a nonevaluability rate of less than 20%, we projected an accrual of 30 patients. In analysis of *EGFR* mutation in serum samples, the categorical variables were compared using the Fisher's exact test. A value of $p < 0.05$ was considered significant. The statistical analyses were performed using the StatView software package, version 5.0 (SAS Institute, Inc., Cary, NC).

## RESULTS

### Patients

From October of 2002 to August of 2003, 30 patients were enrolled into the study. Patient characteristics are summarized in Table 1. The most common histologic subtype was adenocarcinoma (25 patients [83.3%]). Three patients had undergone surgery and three had received radiotherapy to bone or brain metastases. Twenty patients were current or previous smokers. Twenty-six patients (86.7%) had good PS (0–1) and 86.7% of enrolled patients had stage IV disease. A total of 43 sites of metastatic lesions in 26 patients were diagnosed. Thirteen of the 26 patients had more than one metastatic lesion. All four patients with stage IIIb disease had pleural effusion and were ineligible for radiotherapy.

### Efficacy

All patients were assessable for tumor response (Table 2). Complete response was not observed. Ten patients achieved PR, nine had SD as their best response, and 11 patients had progressive disease (PD). The objective response rate was 33.3% (95% confidence interval, 16.2–49.8%) and the disease control rate was 63.3% (95% confidence interval, 46.0–80.5%). All responders had adenocarcinoma. Of the responders, four were male patients and six were female patients. None of the prognostic factors such as gender (male versus female), PS (0–1 versus 2), smoking (never-smoker versus smoker), histology (adenocarcinoma versus nonadenocarcinoma), clinical stage (IIIb versus IV), and prior treatment (yes versus no) was significantly associated with tumor responses (Table 2). Disease control was observed in 19 patients (eight men and 11 women). A significantly higher disease control rate was observed in female patients ($p = 0.018$) and nonsmokers ($p = 0.049$). The other factors did not affect the disease control rate (Table 2).

TABLE 1. Patient Characteristics

| Characteristic | Value |
|---|---|
| No. of patients | 30 |
| Age (yr) | |
| Median | 64 |
| Range | 44–87 |
| Gender | |
| Male | 18 |
| Female | 12 |
| Histology | |
| Adenocarcinoma | 25 |
| Squamous-cell carcinoma | 3 |
| Large-cell carcinoma | 2 |
| Stage | |
| IIIB | 4 |
| IV | 26 |
| Metastatic sites | |
| Pulmonary | 16 |
| Bone | 12 |
| Brain | 11 |
| Others | 4 |
| ECOG performance status | |
| 0 | 20 |
| 1 | 6 |
| 2 | 4 |
| Prior treatment | |
| Yes | 6 |
| Operation | 6 |
| Radiation | 3 |
| No | 24 |
| Smoking | |
| Yes | 20 |
| Pack-years (mean ± SD) | 51 ± 39 |
| No | 10 |

ECOG, Eastern Cooperative Oncology Group.

TABLE 2. Response to Gefitinib Monotherapy and Prognostic Factors*

| | No. | PR | SD | PD | RR (%) | p Value | DCR (%) | p Value |
|---|---|---|---|---|---|---|---|---|
| Total | 30 | 10 | 9 | 11 | 33.3 | | 63.3 | |
| Prognostic factors | | | | | | | | |
| Gender | | | | | | | | |
| Male | 18 | 4 | 4 | 10 | 22.2 | 0.14 | 44.4 | 0.018 |
| Female | 12 | 6 | 5 | 1 | 50.0 | | 91.7 | |
| Smoking habit | | | | | | | | |
| Smoker | 20 | 5 | 5 | 10 | 25 | 0.231 | 50 | 0.049 |
| Nonsmoker | 10 | 5 | 4 | 1 | 50 | | 90 | |
| Histologic type | | | | | | | | |
| Adenocarcinoma | 25 | 10 | 8 | 7 | 40 | 0.139 | 72 | 0.327 |
| Nonadenocarcinoma | 5 | 0 | 2 | 3 | 0 | | 40 | |
| PS | | | | | | | | |
| 0 1 | 26 | 8 | 8 | 10 | 30.8 | 0.584 | 61.5 | 0.999 |
| 2 | 4 | 2 | 1 | 1 | 50 | | 75 | |
| Clinical stage | | | | | | | | |
| IIIb | 4 | 2 | 1 | 1 | 50 | 0.584 | 75 | 0.999 |
| IV | 26 | 8 | 8 | 19 | 31 | | 62 | |
| Prior treatment | | | | | | | | |
| Yes | 24 | 9 | 5 | 10 | 37.5 | 0.999 | 58.3 | 0.215 |
| No | 6 | 1 | 4 | 1 | 16.7 | | 83.3 | |

*RR and DCR were compared between prognostic factors using Fisher's exact test.
*PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control rate.

## TTP and OS

At a median follow-up of 12 months, 20 patients had died and 26 patients were refractory or had become resistant to gefitinib monotherapy. Median TTP was 3.3 months (range, 0.3–19.6 months) and median OS was 10 months (range, 1.7–21.4 months) (Figure 1. Duration of response for patients with partial response was 5.8 months. OS and TTP were not affected by histologic type, smoking, PS, stage, or prior treatment. However, there was a significant difference in survival in gender (median survival time, >12 months in female patients versus 7.7 months in male patients; log-rank test, $p < 0.04$; Wilcoxon test, $p < 0.04$).

## Tolerability

Table 3 shows drug-related adverse events. Twenty-six patients (86.7%) experienced drug-related adverse events, most of which were mild. Frequent adverse events included diarrhea, skin rash, and elevated transaminases. Twenty-two patients experienced skin toxicities, such as acne, pruritus, and rash. Grade 3 skin toxicities were observed in two patients, but these resolved spontaneously during treatment. Diarrhea was observed in 12 patients (40.0%) and was controlled with antidiarrheal agents such as loperamide. One patient developed grade 3 diarrhea, which required temporal interruption of therapy. Two patients developed drug-related pneumonitis; both were treated with steroid therapy, antibiotics, and oxygen inhalation and recovered within a few weeks. These patients were smokers and had not received thoracic radiotherapy. No patients experienced hematologic toxicities.

## Postgefitinib Treatment

Twenty-five patients became resistant or were refractory to gefitinib monotherapy. Eight of these patients received neither chemotherapy nor radiotherapy because of deterioration of PS in four patients and withdrawal of informed consent to chemotherapy in three patients. One patient underwent palliative surgery and two received radiotherapy for symptomatic brain metastases. Fifteen patients received chemotherapy as postgefitinib treatment (platinum-based chemotherapy in 14 patients and vinorelbine monotherapy in one patient). Five patients achieved PR and four showed SD by the second-line chemotherapy.

## EGFR Mutations in Tumor Samples

Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of exons 18, 19, and 21 in *EGFR* was performed in 12 of 20 samples under the same PCR conditions. *EGFR* mutations were detected in four tumor

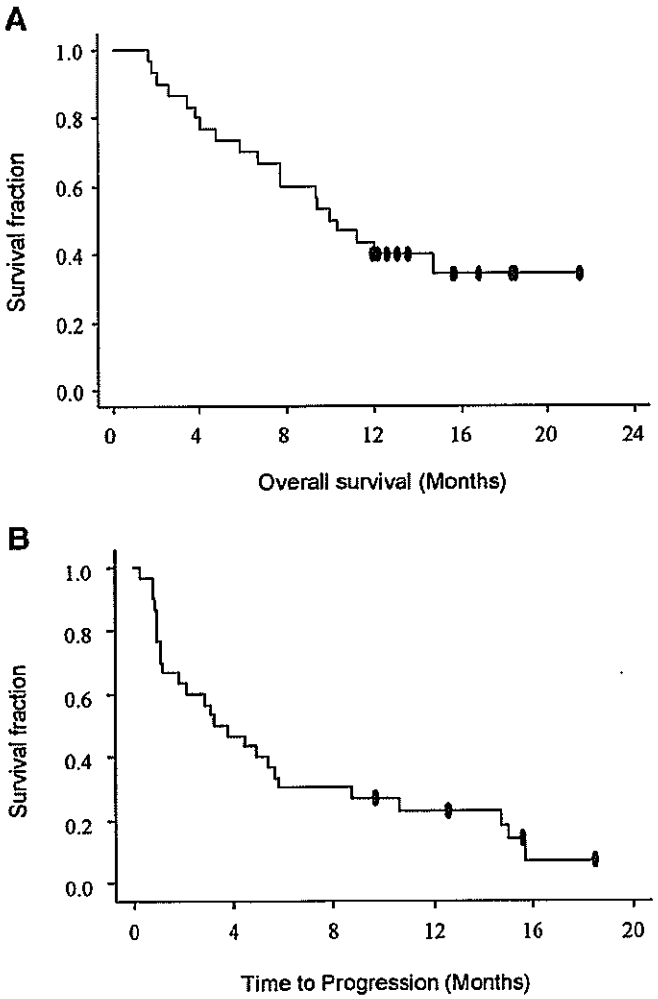


FIGURE 1. Kaplan-Meier curve showing (*A*) overall survival and (*B*) time to progression in all patients.

TABLE 3. Drug-Related Adverse Events

| | NCI-CTC Grade | No. of Patients | % |
|---|---|---|---|
| Diarrhea | 1 | 8 | 26.7 |
| | 2 | 3 | 10.0 |
| | 3 | 1 | 3.3 |
| Nausea | 1 | 8 | 26.7 |
| | 2 | 2 | 6.7 |
| | 3 | 0 | 0.0 |
| Vomiting | 1 | 2 | 6.7 |
| | 2 | 0 | 0.0 |
| | 3 | 0 | 0.0 |
| Skin toxicity | 1 | 15 | 50.0 |
| | 2 | 5 | 16.7 |
| | 3 | 2 | 6.7 |
| Elevation of transaminases | 1 | 4 | 13.3 |
| | 2 | 1 | 3.3 |
| | 3 | 2 | 6.7 |
| Pneumonitis | 1 | 0 | 0.0 |
| | 2 | 0 | 0.0 |
| | 3 | 2 | 6.7 |

NCI-CTC, National Cancer Institute Common Toxicity Criteria.

samples (33.3%). Three of them had a 15-base pair deletion (E746_A750del) in exon 19. Another of them had L858R in exon 21. The histologic types in patients with *EGFR* mutations were adenocarcinoma in three and large-cell carcinoma in one. All patients with E746_A750del in tumor samples had adenocarcinoma. The responses to gefitinib in these four patients were PR in two, SD in one, and PD in one. There were no responders among nine patients without an *EGFR* gene mutation.

## EGFR Mutations in Serum Samples

The serum DNA in serum samples from 27 NSCLC patients was examined. Serum DNA was detected in all 54 samples at concentrations of up to 1720 ng/ml.

Exon 19 of *EGFR* in pretreatment serum samples obtained from 21 of 27 patients (77%) was detected (Figure 2 *A*). The lower band was also detected in 10 of 27 (37%) pretreatment serum samples. Sequencing of the PCR products confirmed that the upper and lower bands corresponded to wild-type and E746_A750del, respectively (Figure 2 *B*). No point mutation in exon 18, 19, or 21 was detected in the PCR products from serum samples. Wild-type *EGFR* was detected in all 10 of the deletion-positive cases. The pattern of bands was reproducible when using another primer set.[13]

When compared according to histologic type, E746_A750del was detected in eight of 25 (32%) cases of adenocarcinoma, in zero of three cases of squamous carcinoma, and in two of two cases of large-cell carcinoma (Table 4). In contrast, the serum *EGFR* status was not correlated statistically with either the clinical response, the gender, or the recorded adverse effects (Table 5).

In serum samples obtained after the initiation of gefitinib treatment, 19 of 27 (70%) cases were wild-type-positive and 14 of 27 (52%) cases were deletion-positive (Figure 2 *C*). In the posttreatment serum samples, E746_A750del was more frequently observed. Furthermore, the deletional mutant of *EGFR* was significantly more frequently observed in samples from patients who showed a PR or SD (12 of 16 cases [75%]) than in samples from patients with PD (two of 11 cases [18%]) ($p = 0.0063$, Fisher's exact test) (Table 6). The deletional mutant *EGFR* was more frequently detected in female patients (six of nine cases [67%]) than in male patients (eight of 18 cases [44%]), but this difference was not significant (Table 6). No correlations were seen statistically between the presence of mutation and the adverse effects.

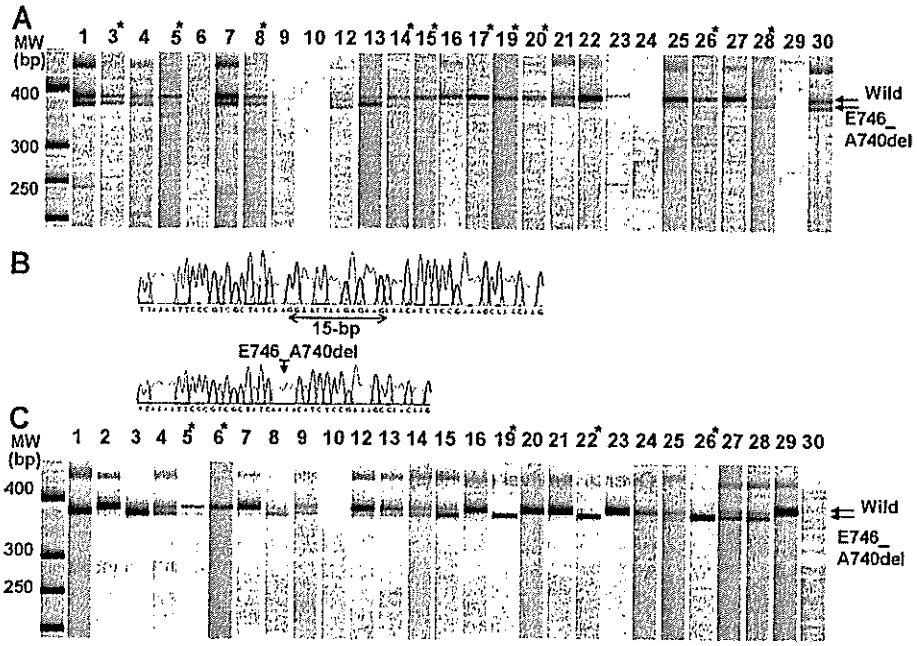


FIGURE 2. (*A*) Detection of genomic *EGFR* in the serum of pretreatment patients. (*B*) The sequences of the PCR products from patient 19 (days 0 and 14) are shown. (*C*) PCR of the serum samples obtained on day 14. Serum-derived genomic DNA PCR was performed. Exon 19 of *EGFR* in serum obtained from the patients was amplified by PCR, and the products were detected using a Bioanalyzer. A second round of PCR (20 cycles) was performed when no band was detected in the first round of PCR (30 cycles). Row numbers indicate the patient number. *Band detected in the first round of PCR.

TABLE 4. Frequency of Serum *EGFR* in Lung Cancer Patients According to Histology and Response to Gefitinib*

| | Pre | | Post | |
|---|---|---|---|---|
| | Wild | Deletion | Wild | Deletion |
| Adenocarcinoma | 18/23 | 8/23 | 15/22 | 13/22 |
| Squamous-cell carcinoma | 1/2 | 0/2 | 3/3 | 0/3 |
| Large-cell carcinoma | 2/2 | 2/2 | 1/2 | 1/2 |

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking.

TABLE 5. Frequency of Serum *EGFR* in Lung Cancer Patients According Response to Gefitinib and Gender: Detection of Deletion-Type Mutation on Day 0*

| | + | − | *p* Value |
|---|---|---|---|
| Response | | | |
| PR/SD | 8 | 9 | |
| PD | 2 | 8 | 0.2305 |
| Gender | | | |
| Male | 5 | 12 | |
| Female | 5 | 5 | 0.4153 |

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking. SD, stable disease; PD, progressive disease; PR, partial response; +, deletion-positive; −, wild-type.

## Comparison of *EGFR* Mutation Status between Tumor Samples and Serum Samples

Pairs of tumor samples and serum samples were obtained from 12 patients retrospectively (Table 7). The *EGFR* mutation status in the tumors was consistent with those in serum of seven of 12 of the paired samples. Among the other five patients, *EGFR* mutation was negative in the tumor and positive in the serum in four patients, and in the other patient it was positive in the tumor and negative in the serum, from whose tumor sample L858R was detected.

TABLE 6. Frequency of Serum *EGFR* in Lung Cancer Patients According to Response to Gefitinib and Gender: Detection of Deletion-Type Mutation on Day 14*

| | + | − | *p* Value |
|---|---|---|---|
| Response | | | |
| PR/SD | 12 | 4 | |
| PD | 2 | 9 | 0.0063 |
| Gender | | | |
| Male | 8 | 10 | |
| Female | 6 | 3 | 0.4197 |

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking. SD, stable disease; PD, progressive disease; PR, partial response; +, deletion-positive; −, wild-type.

## DISCUSSION

The overall response of 33.3% in this phase II study was comparable not only to that achieved in Japanese population enrolled in the IDEAL-1 trial (27.5%)[7] but also to a retrospective analysis conducted of patients in Japan.[22] Gefitinib monotherapy appeared to be equally effective in patients with chemotherapy-naive NSCLC and in patients with pretreated NSCLC.

Drug-related adverse events were generally mild compared with cytotoxic chemotherapy. Grade 3 pulmonary toxicities were observed in two patients. In this study, the

TABLE 7. *EGFR* Mutation Status in Tumor Samples and Serum Samples*

| | | | | | EGFR Mutation Status | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Serum Samples | | | |
| | | | | | Pre | | Post | |
| No. | Gender | Histology | Response | Tumor Sample | Wild | Mutation | Wild | Mutation |
| 43 | M | Large | SD | Wild | + | + | | + |
| 45 | M | SCC | PD | Wild | ND | ND | + | - |
| 52 | F | SCC | PD | Wild | + | | + | - |
| 53 | M | Adeno | PD | Wild | - | - | + | |
| 55 | M | Adeno | PR | L858R | + | + | - | - |
| 57 | F | Adeno | SD | Wild | | - | + | + |
| 61 | M | Large | PD | E746-A750 del | + | + | + | - |
| 64 | M | Adeno | PD | Wild | + | - | + | - |
| 70 | M | Adeno | PD | Wild | + | + | + | - |
| 72 | M | Adeno | SD | E746-A750 del | + | - | - | + |
| 75 | F | Adeno | PR | E746-A750 del | + | + | + | + |
| 77 | M | Adeno | PD | Wild | + | - | + | + |

*Pairs of both tumor samples and serum samples were obtained from 12 patients. M, male; F, female; SD, stable disease; PD, progressive disease; PR, partial response; SCC, squamous-cell carcinoma; Adeno, adenocarcinoma; Large, large-cell carcinoma; ND, not determined.

incidence of drug-related pneumonitis was 6.7% and was comparable to results of other studies.[23,24] Therefore, gefitinib monotherapy as a first-line treatment appears to be equally tolerable as a second-line treatment.

Thirteen of 22 patients who became resistant or were refractory to gefitinib monotherapy received salvage chemotherapy. The objective response rate was 30.8%, comparable to that of first-line chemotherapy. These results suggest that cancer cell populations that are sensitive to gefitinib might not be identical to those sensitive to chemotherapeutic drugs such as platinum agents or taxanes.

Somatic mutations in the tyrosine kinase domain of the *EGFR* gene were reported, and these mutations induced increased activity of *EGFR* and sensitivity to gefitinib in vitro and the predictive factor of response to gefitinib.[13,14] We evaluated *EGFR* gene status in 13 tumor samples and detected *EGFR* gene mutation in four tumors. Objective responses were achieved in two patients, but one patient showed PD whose tumor had a 15 base pair deletion mutation in exon 19. This suggested that response to gefitinib may not be determined by *EGFR* mutation in exon 19 or 21, and other mechanisms may relate to gefitinib resistance.

The detection of EGFR mutation from serum samples was carried out as a correlative study. These results provided us two major findings: (1) E746_A750del was detectable in serum sample obtained from NSCLC patients; and (2) E746_A750del was frequently observed in posttreatment serum samples obtained from the PR and SD patients.

It may be explained that DNA derived from destructive tumor cells that have responded to gefitinib may be more frequently observed in the circulating blood. Previous reports regarding detection of mutations in serum did not elucidate the changes in mutation status during treatment. We would like to do this in the next experiments to confirm our speculation. Our hypothesis is that serum detection of *EGFR* mutation will be a convenient means of predicting the sensitivity to gefitinib, although we could only demonstrate the feasibility of the *EGFR* mutation in serum in this report. We need to develop a highly sensitive methodology to improve the predictability of this assay.

In comparison of the mutation status of *EGFR* in actual tumors with serum DNA obtained from the same patients before treatment, 70% of patients who had sequence data obtained from both serum and tumor samples were conforming. Esteller et al. reported detection of aberrant promoter hypermethylation of tumor suppressor genes (*p16*, *DAP*, *GSTP1*, and *MGMT*) in serum DNA obtained from NSCLC patients and demonstrated that 73% of serum samples showed abnormal methylated DNA in the patients with the methylated primary tumors.[19] Another report investigating a point mutation of the *p53* gene and hypermethylation of *p16* in plasma DNA from breast cancer patients demonstrated that 66% of the patients with at least one molecular event in tumor DNA had some alteration in plasma DNA.[25] We believe that the sensitivity of our assay is equivalently sensitive to those of these previous reports.

## CONCLUSION

In conclusion, 250 mg of oral gefitinib monotherapy as a first-line treatment produces obvious antitumor activity, with acceptable toxicities. Oral gefitinib monotherapy as a first-line treatment merits investigation in further clinical trials. Using serum samples from NSCLC patients, the *EGFR* mutation was detected. The detection of E746_A750del in the serum of untreated patients was not a predictor of gefitinib response in this study. However, further prospective studies using serum samples may be necessary to confirm this con-

clusion. The presence of *EGFR* mutation in serum may be a useful biomarker for monitoring gefitinib response.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Parkin DM, Bray F, Ferlay J, et al. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153–156.
2. Roskoski R Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun* 2004;319:1–11.
3. Scagliotti GV, Selvaggi G, Novello S, et al. The biology of epidermal growth factor receptor in lung cancer. *Clin Cancer Res* 2004; 10: 4227s–4232s.
4. Rusch V, Baselga J, Cordon-Cardo C, et al. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res* 1993;53: 2379–2385.
5. Rusch V, Klimstra D, Venkatraman E, et al. Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression. *Clin Cancer Res* 1997;3:515–522.
6. Fontanini G, De Laurentiis M, Vignati S, et al. Evaluation of epidermal growth factor-related growth factors and receptors and of neoangiogenesis in completely resected stage I-IIIA non-small-cell lung cancer: amphiregulin and microvessel count are independent prognostic indicators of survival. *Clin Cancer Res* 1998;4:241–249.
7. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21:2237–2246.
8. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 2003;290:2149–2158.
9. Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol* 2004;22:785–794.
10. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol* 2004;22:777–784.
11. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 2006;366: 1527–1537.
12. Johnson DH. Gefitinib (Iressa) trials in non-small cell lung cancer. *Lung Cancer* 2003; 41 (Suppl 1): S23–S28.
13. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
14. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304: 1497–1500.
15. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancer from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;101:13306–13311.
16. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2004;97:339–346.
17. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 2006;23:2493–2501.
18. Sanchez-Cespedes M, Monzo M, Rosell R, et al. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. *Ann Oncol* 1998;9:113–116.
19. Esteller M, Sanchez-Cespedes M, Rosell R, et al. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;59:67–70.
20. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
21. Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials* 1989;10:1–10.
22. Takano T, Ohe Y, Kusumoto M, et al. Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. *Lung Cancer* 2004;45:93–104.
23. Takeda K, Yamamoto N. An epidemiological survey for interstitial lung disease induced by gefitinib in patients with advanced non-small cell lung cancer. *Lung Cancer* 2003;41:S250.
24. Hotta K, Harita S, Bessho A, et al. Interstitial lung disease (ILD) during gefitinib treatment in Japanese patients with non-small cell lung cancer (NSCLC): Okayama Lung Cancer Study Group. *Proc Am Soc Clin Oncol* 2004;23:629.
25. Silva JM, Dominguez G, Garcia JM, et al. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. *Cancer Res* 1999;59:3251–3256.

 

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# SYNTHESIS AND BIOLOGICAL PROPERTIES OF NEW PHOSMIDOSINE ANALOGS HAVING AN *N*-ACYLSULFAMATE LINKAGE

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□ *A new phosmidosine analog* **10**, *in which the proline and 8-oxoadenosine moieties were linked by an* N*-acyl sulfamate linkage, was successfully synthesized by the sulfamoylation of an 8-oxoadenosine derivative* **5** *followed by coupling with an* L*-proline derivative* **8**. *An* L*-alanine-substituted derivative* **13** *and its derivative* **14** *without the alanyl residue were also synthesized. The morphological reversion activity of these synthetic compounds in v-src$^{ts}$ NRK cells and their antitumor activity in L1210 and KB cells were studied. As the result, neither* L*-proline- nor* L*-alanine-substituted phosmidosine analogs* **10** *and* **13** *showed any antitumor activity. Contrary to these results, the derivative* **14** *lacking the amino acid residue showed potent antitumor activities against cancer cells.*

**Keywords** *N*-acyl sulfamate linkage; Phosmidosine analogs; Morphological reversion activity; Antitumor activity

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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## INTRODUCTION

A number of artificial aminoacyl adenylate derivatives having an *N*-acylsulfamate linkage have been synthesized and their biological properties have been studied.[1–4] Alanyl-, arginyl-, prolyl-, and asparaginyl adenylate analogs were synthesized. The chemical stability of the *N*-acylsulfamate linkage of aminoacyl adenylate derivatives is higher than that of the corresponding *N*-acylphosphoramidate linkage under physiological conditions. Therefore, a series of aminoacyl adenylate analogs containing *N*-acylsulfamate linkages have been used as aminoacyl-tRNA synthetase inhibitors.[1–3] Nucleocidin, which has an *N*-acylsulfamate linkage lacking the aminoacyl residue, is known to be highly toxic and to act as a highly potent inhibitor of protein synthesis.[4,5] Ascamycin, which was isolated from *Xanthomonas spp.* in 1984,[6] is a nucleoside derivative possessing an *N*-acylsulfamate linkage and a 2-chloroadenine residue as the nucleobase. Isono et al. reported the biological properties of ascamycin and its analogs substituted with other amino acids, showing that these compounds have highly potent antibacterial activities.[7] They also reported that a dealanylascamycin called AT-256, which was produced by Xc-aminopeptidase-promoted hydrolysis of ascamycin,[8] inhibited protein synthesis. Aminoacyl adenylate derivatives having an *N*-acylphosphoramide linkage have also been studied and their chemical and biological properties have been clarified. The P–N bond of the *N*-acylphosphoramide linkage is more stable than the corresponding P–O bond of an *O*-acylphosphoramide linkage and these modified nucleosides showed antitumor activities. A naturally occurring antibiotic, phosmidosine, has proved to possess potent antitumor activities against various human cancer cells.[9–11] McCloskey reported that phosmidosine was decomposed by treatment with 0.2 M NaOH to produce a proline moiety-lacking compound and rearranged compounds.[12] Recently, we have studied the synthesis of phosmidosine and its analogs.[13–17] In our continuous studies of the structure-activity relationship of a series of phosmidosine derivatives, we found that the 8-oxoadenine base and the proline moiety were essential for inhibition of the cancer cell growth.[15] These results prompted us to synthesize new phosmidosine analogs having an *N*-acylsulfamate linkage.

## RESULTS AND DISCUSSION

A general procedure for construction of *N*-acylsulfamoyl linkage has been developed to obtain a series of aminoacyl adenylate derivatives containing an *N*-acylsulfamoyl linkage.[1–4] Therefore, we applied this method to the synthesis of our new phosmidosine analogs.

Thus, the *O*-selective reaction of an appropriately protected 8-oxoadenosine derivative 5 with sulfamoyl chloride,[2,18] which was prepared from chlorosulfonyl isocyanate and formic acid, was studied. As the result,

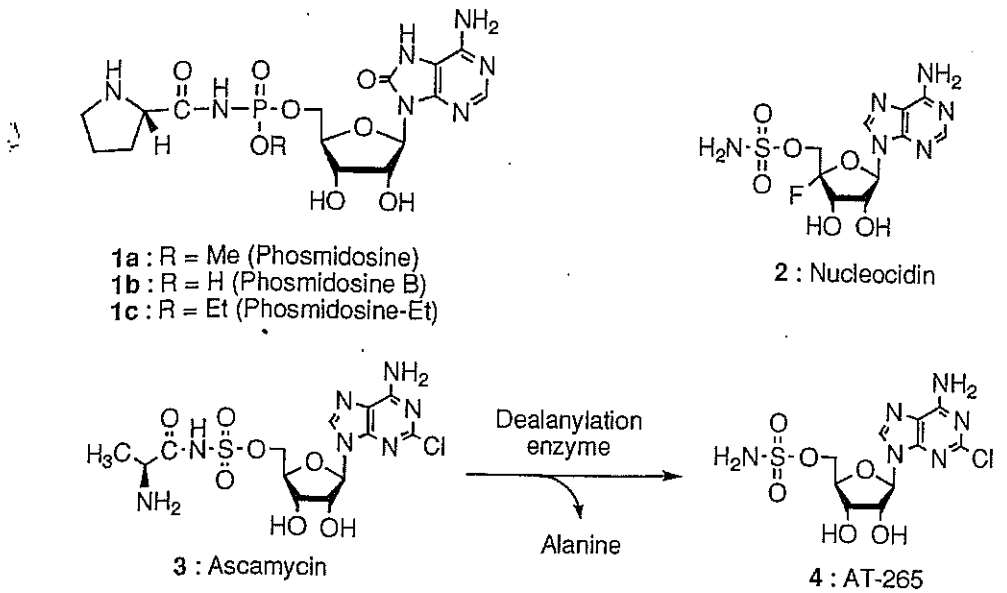


**FIGURE 1** Structure of several aminoacyl adenylate derivatives.

the desired sulfamoylation proceeded to afford the 5′-*O*-sulfamoyl-8-oxoadenosine derivative **6**. In this reaction, no reactions occurred on the 7-position or the 6-amino group of the 8-oxoadenine moiety. In an attempt to obtain an *N*-acyl sufamate derivative **9**, an L-proline derivative was activated by treatment with *N*, *N*′-carbonyldiimidazole and the resulting acylimidazole derivative **7** was allowed to react with **6**. However, no prolylated compounds were obtained. In contrast to this result, when the *O*-succsinyl-L-proline derivative **8**[2] was used, the reaction gave the desired product **9** in a moderate yield. The yield of **9** was increased to 80% by the choice of

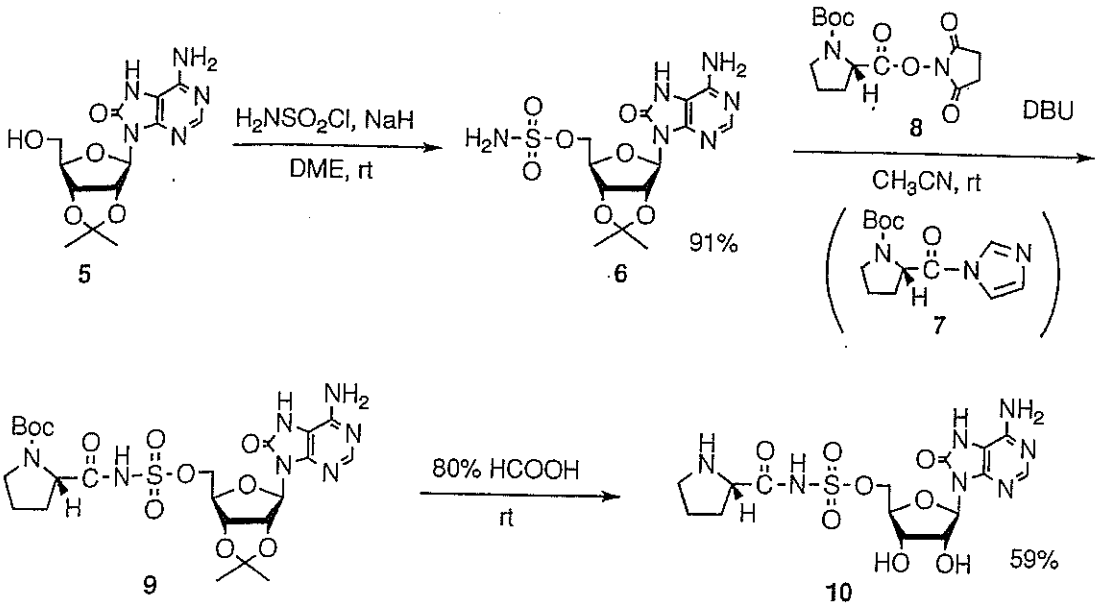


**FIGURE 2** Synthesis of phosmidosine analogs.

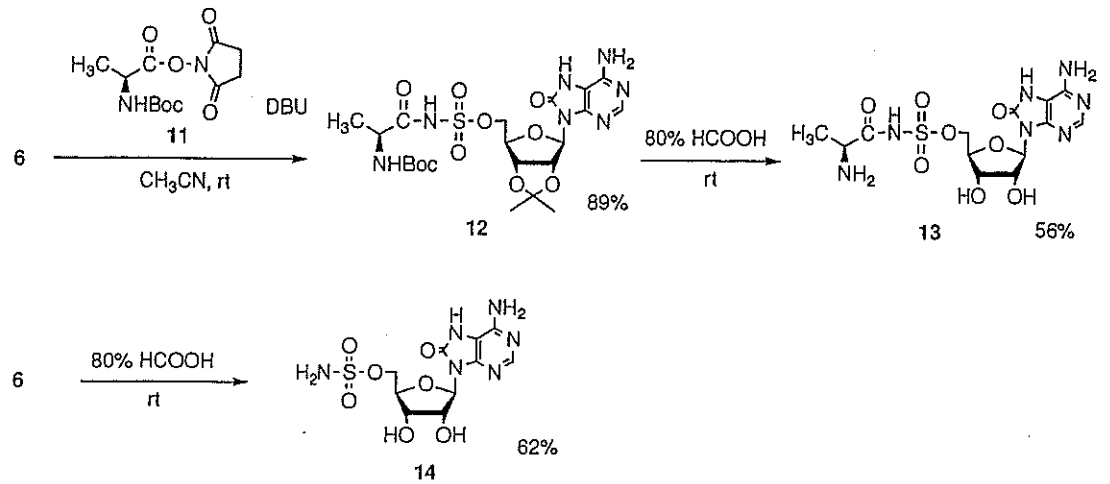


**FIGURE 3** Synthesis of ascamycin mimics.

acetonitrile as the solvent. The product was treated with 80% formic acid for 12 h to give the product **10** as an amorphous white solid in 59% yield.

In a similar manner, we also synthesized an ascamycin mimic **13**, which was obtained by the reaction of **6** with the ester **11** followed by acidic treatment of the product **12**. The compound **14**, which does not have the alanyl residue, was synthesized by treatment of **6** with 80% HCOOH.

The morphological reversion activity of these synthetic compounds in v-src$^{ts}$NRK cells and their antitumor acttivity in L1210 and KB cells were studied. These results are shown in Table 1.

**TABLE 1** The Biological Properties of Compounds **1c**, **10**, **13**, and **14**

| | Morphological reversion activity ($\mu$g/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Aminoacyl nuclesides | 100 | 30 | 10 | 3 | 1 | 0.3 | 0.1 |
| Morphological reversion activity of phosmidosine analogs in v-src$^{ts}$ NRK cells | | | | | | | |
| L-Pro-Sulfamoyl-8-oxoA : **10** | − | − | − | − | − | − | − |
| H-Sulfamoyl-8-oxoA : **14** | +++ | +++ | +++ | +++ | + | − | − |
| Phosmidosine-Et : **1C** | nt | nt | +++ | +++ | + | nt | nt |

| | L1210 $\mu$M | KB $\mu$M |
|---|---|---|
| $IC_{50}$ Values of phosmidosine analogs in L1210 and KB cells | | |
| L-Pro-Sulfamoyl-8-oxoA : **10** | 218< | 218< |
| L-Ala-Sulfamoyl-8-oxoA : **13** | 231< | 231< |
| H-Sulfamoyl-8-oxoA : **14** | 0.91 | 4.86 |
| Phosmidosine-Et : **1C** | 3.62 | 3.44 |

+++:More than 75% of cancer cells were morphologically reversed.
++:25–75% of cancer cells were morphologically reversed.
+:ca. 25% oc cancer cells were morphologically reversed.
−:no activity; nt: not tested.

As shown in Table 1, the phosmidosine ethyl ester **1c** was used as a control sample. In the morphological reversion activity assay, the L-proline-substituted phosmidosine analog **10** did not show any morphological reversion activities. However, compound **14** showed morphological reversion activity at a low concentration. Even when the concentration was 3 $\mu$g/ml, morphological reversion activity was significantly observed. Next, we studied the antitumor activity of these compounds against L1210 and KB cells. The results were similar to those obtained in the case of the morphological reversion activity assay. Only compound **14** inhibited the growth of L1210 and KB cells. The structure of compound **14** is similar to those of nucleocidin **2** and AT-265 **4**.

In conclusion, we have successfully synthesized a new phosmidosine analog having an *N*-acylsulfamoyl linkage. An L-alanine-substituted derivative **10** and its derivative **14** were also synthesized in a similar manner. The biological properties of these new compounds were studied and it was found that 5′-*O*-sulfamoyl-8-oxo-adenosine **14** showed potent activity against human cancer cells. Further studies on the mode of action of these compounds are in progress.

## EXPERIMENTAL

$^1$H and $^{13}$C NMR spectra were obtained at 270 and 68 MHz, respectively. The chemical shifts were measured from DMSO-$d_6$ (2.49 ppm) and 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid sodium salt (TSP-$d_4$) (0 ppm) for $^1$H NMR and from DMSO-$d_6$ (39.7 ppm) for $^{13}$C NMR. Column chromatography was performed with silica gel C-200. Reverse-phase column chromatography was performed by use of 37-55 $\mu$m $C_{18}$ particle. Mass spectra were measured by use of an ESI-mass spectrophotometer. *In vitro* analysis of the antitumor activity in cancer cell lines was carried out by the literature method reported by Carmichael[19] and us.[15] The morphological reversion activity test was conducted according to the literature method.[10] Compound **5** was synthesized according to our previous paper.[15]

*2′,3′-O-Isopropylidene-5′-O-sulfamoyl-8-oxoadenosine* (**6**). Under argon atmosphere, compound **5** (1.51 g, 5 mmol) was coevaporated three times with dry pyridine and dissolved in dry DME (25 ml). To this DME solution was added sodium hydrate (60%, 480 mg, 12 mmol), and the mixture was stirred at 0°C for 30 min. A DME (25 ml) solution of sulfamoyl chloride (1.16 g, 10 mmol) was added to the mixture. After being stirred at room temperature for 10 h, the mixture was quenched by addition of 20 ml of methanol and evaporated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$:methanol = 95:5, v/v) to give compound **6** (1.79 g, 89%): $^1$H NMR (DMSO-$d_6$) $\delta$ 1.31 (3H, s), 1.51 (3H, s), 4.05–4.32 (3H, m), 4.98 (1H, d, $J_{2',3'} = 6.3$ Hz), 5.39 (1H, d, $J_{2',3'} = 6.3$ Hz), 5.93 (1H, s), 7.15 (2H, bs), 7.54 (2H, bs), 8.17 (1H, s), 10.94 (1H, bs); $^{13}$C NMR